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COLONY-LEVEL IMPACTS OF IMMUNE RESPONSIVENESS IN HONEY BEES, *APIS MELLIFERA*

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Abstract.—Social insects have evolved both communal and individual traits that reduce the impacts of their numerous parasites and pathogens. Among the individual traits, innate-immune responses have the potential to reduce both individual mortality and the spread of pathogens among colony members. An understanding of the costs and benefits of such responses can provide a more complete understanding of a primary risk of social life, horizontal disease transmission among colony members. Here we assess the impacts of individual immunity on colony-level disease in honey bee (*Apis mellifera*) colonies following exposure to an important bacterial pathogen (*Paenibacillus larvae* subsp. *larvae*, cause of the disease American foulbrood). Colony-level disease rates were negatively correlated with the immune responsiveness of colony members, as assessed by larval transcript levels for the gene encoding the antibacterial peptide abaecin. Concomitantly, colonies whose members mounted a stronger abaecin response showed significantly lower productivity, indicating a colony-level cost to this immune response. The results show considerable variation across colonies in an immune trait important for survival, and point toward a significant trade-off between this trait and colony productivity.

Key words.—American foulbrood, antimicrobial peptide, ecological immunity, innate immunity, life-history trade-off, *Paenibacillus larvae*.

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Like many eukaryotes, insects rely on innate-immune responses in their battles against parasites and pathogens. These responses take diverse forms, ranging from the ability to isolate alien organisms by phagocytosis or melanization to the upregulation of a diverse suite of antimicrobial peptides (Brennan and Anderson 2004; Irving et al. 2004). Both phenotypic studies (Cotter and Wilson 2002; Lambrechts et al. 2004) and sequence-level analyses (Lazzaro et al. 2004) indicate widespread heritable variation in the magnitude and character of insect immune responses toward natural pathogens. Such variation is puzzling, because the high fitness costs of disease are predicted to select for reduced levels of genetic variation in immune traits. Two primary hypotheses have been put forth to explain this puzzle. First, host-parasite dynamics such as conditional or frequency-dependent selection exerted by pathogens on insect hosts could favor allelic variation within host populations for genes having an impact on immunity (Schmid-Hempel 2004). Second, host-level trade-offs related to the cost of mounting an immune response (Armitage et al. 2003; Cotter et al. 2004) might lead to divergent strategies in host populations. Some individuals could rely on a strong immune response, presumably with a concomitant energetic cost, whereas others might forego immunity, thereby achieving higher reproductive success when parasites are absent.

Social insects face especially high risks from parasites and pathogens, due to crowded living conditions and the potential that closely related nestmates will share the same vulnerabilities against specific pathogens (Schmid-Hempel 1998; cf. Kraus and Page 1998). Among the social insects, honey bees have an exceptionally diverse set of parasites and pathogens and are known to suffer both reduced productivity and colony failure due to disease (Morse and Flottum 1997). Infections by the primary bacterial pathogen of honey bees, the gram-positive species *Paenibacillus larvae* subsp. *larvae* (cause of American foulbrood disease) appear to be mitigated by both hygienic behaviors among adults (Spivak and Reuter 2001) and larval resistance traits (Rothenbuhler and Thompson 1956; Evans 2004). Resistance by larval bees to *P. l. larvae* appears to have a genetic component, as shown by subfamily variation in survival (Palmer and Oldroyd 2003) and variation across individuals in their immune responses to bacterial infection (Evans 2004). Here we show strong relationships between immune-gene transcription, disease at the colony level, and colony productivity. The results suggest that an innate immune response is important in reducing disease, but that such responses come at the expense of reducing colony productivity.

MATERIALS AND METHODS

Colony Establishment and Disease Challenge

Colonies were established in April 2002 in two adjacent apiaries near the Bee Research Laboratory (Beltsville, MD). Each colony was formed by placing 1.2 kg of worker bees and a queen representing a commercial ''Italian'' lineage from Georgia, United States (*Apis mellifera*) into standard (Langstroth) hives. Following establishment, each colony was inoculated twice (5/1/02 and 6/4/02) with spores from a field isolate of *P. l. larvae*, the causative agent of American foulbrood disease (AFB; Shimanuki 1997). Inoculations consisted of spraying approximately 2000 immature bees (eggs, embryos, and first- and second-instar larvae) with a sucrose-water suspension containing about 200 million *P. l. larvae* spores. This inoculant of *P. l. larvae* originated from 100 scales (dried larval remains) collected from four colonies in Beltsville, Maryland, that had AFB the previous year. Spray inoculation is effective at initiating AFB infection in colonies, with about 50% of managed colonies exhibiting AFB disease one month following a single inoculation (24/47 colonies inoculated in 2003; J. Pettis, unpubl. data).

Colony Surveys

Colonies were inspected monthly to assess disease levels and colony growth, and to ensure that all colonies were successfully inoculated. Approximately 90 days after colony establishment, colony growth was measured by removing individual frames and estimating the area of sealed immature brood on each side of all frames using a clear 5×5 cm^2 grid. ''Frames'' were defined as being equivalent to one side of bee larvae on a standard hive (Langstroth) frame. Simultaneously, the level of foulbrood disease was determined by making a visual inspection of all brood (immature larval and pupal honey bees) for evidence of infection. Signs of this disease are unmistakable and include partially uncapped cells, foul odor, and the presence of abnormal brood within cells (Shimanuki and Knox 2000).

Severity of AFB infection was quantified using a modification of a standard scoring method (Hitchcock et al. 1970). Each frame with brood was rated on a 0–3 scale as to AFB infection. A score of zero equals no visible signs of disease; 1, less than 10 cells with visible AFB; 2, 11–100 cells with AFB; and 3, greater than 100 cells with AFB. A composite disease score was generated by summing across all frames for each colony.

Larval Immune-Response Assays

To screen larvae for immune responsiveness, first-instar larvae were challenged by oral doses of the foulbrood bacterium *P. l. larvae*, as described previously (Evans 2004). In total, 256 larvae (16 each from 16 managed field colonies) were challenged with a single isolate of *P. l. larvae* (distinct from that used to challenge field colonies), then reared at 34°C in an incubator at high humidity. After 24 hours, larvae were preserved at −80°C prior to gene-expression analyses.

Twelve larvae from each of these colony samples were assayed for immune-gene expression. Total RNA was extracted from individual larvae using the RNAqueous protocol (Ambion, Austin, TX) in 96-well extraction plates. To minimize plate effects during the expression assays, colony samples were divided across two plates prior to extraction (e.g., each 96-well plate contained six bees each from all 16 colonies). Contaminating DNA was removed with two DNAse I treatments, once during RNA extraction and once immediately prior to cDNA synthesis (Evans 2004). First-strand cDNAs were generated from approximately 2 μg total RNA using a mix of 50 U Superscript II (Invitrogen, Carlsbad, CA), 2 nmol DNTP mix, and a composite of 2 nmol poly dT-18 and 0.1 nmol poly dT(12–18). Synthesis was carried out at 45°C for one hour.

Transcript copies were quantified using real-time PCR in 96-well microtiter trays using specific oligonucleotide primers and an Icycler Real-Time thermal cycler (Bio-Rad, Hercules, CA). Twenty-five μl PCR reaction mixes consisted of one U Taq DNA polymerase with recommended buffer (Roche Applied Science, Indianapolis, IN), 1 mM DNTP mix, 2 mM MgCl2, 0.2 μM of each primer. Transcript levels for a moderately expressed housekeeper gene of honey bees (ribosomal protein S5; (RpS5; Evans and Wheeler 2000; Evans 2004) were used to normalize against variable cDNA levels, while transcript levels for bee genes encoding the antimicrobial peptides abaecin and defensin (Casteels et al. 1990) were used to assess the immune response. Transcripts for two genes from *Paenibacillus larvae*, a spore germination protein (GermSA; GenBank accession no. DQ190842), and the FLiP flagellar biosynthesis protein (GenBank no. DQ190843) were used to assess pathogen levels. Internal sequence probes containing the fluorophores 6-FAM or HEX paired with a Taqman-FRET 3′ quencher (Applied Biosystems, Foster City, CA; Table 1) were used to assess levels of the three honey bee genes, whereas the generic reporter SYBR Green (Invitrogen) was used for the two bacterial genes.

TABLE 1. Oligonucleotide amplification primers and internal probes for quantitative RT-PCR. RPS5 is a control gene used to normalized honey bee RNA levels, abaecin and defensin are honey bee immune genes, and PlGermSA and PlFLiP are genes of the bacterial pathogen. See the text for GenBank accession numbers.

Locus	Primer sequences (5′–3′)
RPS5	F: AATTATTTGGTCGCTGGAATTG
	R: TAACGTCCAGCAGAATGTGGTA
	Pr: 6FAM-GCCGTTAAAGAGAAAAATGCAA
Abaecin	F: CAGCATTCGCATACGTACCA
	R: GACCAGGAAACGTTGGAAAC
	Pr: HEX-TGTACCACAACCAGGACGTC
Defensin	F: TGCGCTGCTAACTGTCTCAG
	R: AATGGCACTTAACCGAAACG
	Pr: 6FAM-TGGGTAAAGCTGGAGGTCAT
PlGermSA	F: CCATTTGCTTCAGGGAAGAG
	R: CAAGCCAGCGTATGCTGTAA
PlFLiP	F: TGCAGTCCAGCCGTACATTA
	R: ATATCATGACCGGAGGCAAC

Real-time PCR reactions using RPS5 and defensin relied on a two-step protocol, 95°C for 3 min followed by 40 cycles of 95°C for 30 sec, and 55°C for 1 min. For abaecin, a three-step program of 40 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, 30 sec was used. For the two bacterial genes, an identical three-step program was used, albeit with a 62°C annealing temperature. Fluorescence was measured repeatedly each cycle during the annealing step. For each sample/primer combination, fluorescence levels were normalized within wells using average fluorescence during cycles 2–10. Threshold cycles were defined as the point when well fluorescence became greater than 10 times the mean standard deviation across all samples. Threshold cycle (C_T) numbers for the immune-peptide genes abaecin and defensin were then subtracted from the RPS5 threshold for each sample prior to statistical analyses. To avoid possible autocorrelation between honey bee transcripts and those of their bacterial pathogens, bacterial transcripts for the germination protein SA and the flagellar synthesis protein were not normalized to RPS5, but were instead normalized to their respective study-wide means.

Data Analyses

Colony-level brood scores and disease scores showed a strong linear relationship ($r^2 = 0.88$, $P < 0.0001$). Accordingly, colony-specific disease estimates were based on residual values from a regression of brood scores on disease scores. Two-way analyses of variance were carried out using these residual values along with productivity (frames of de-

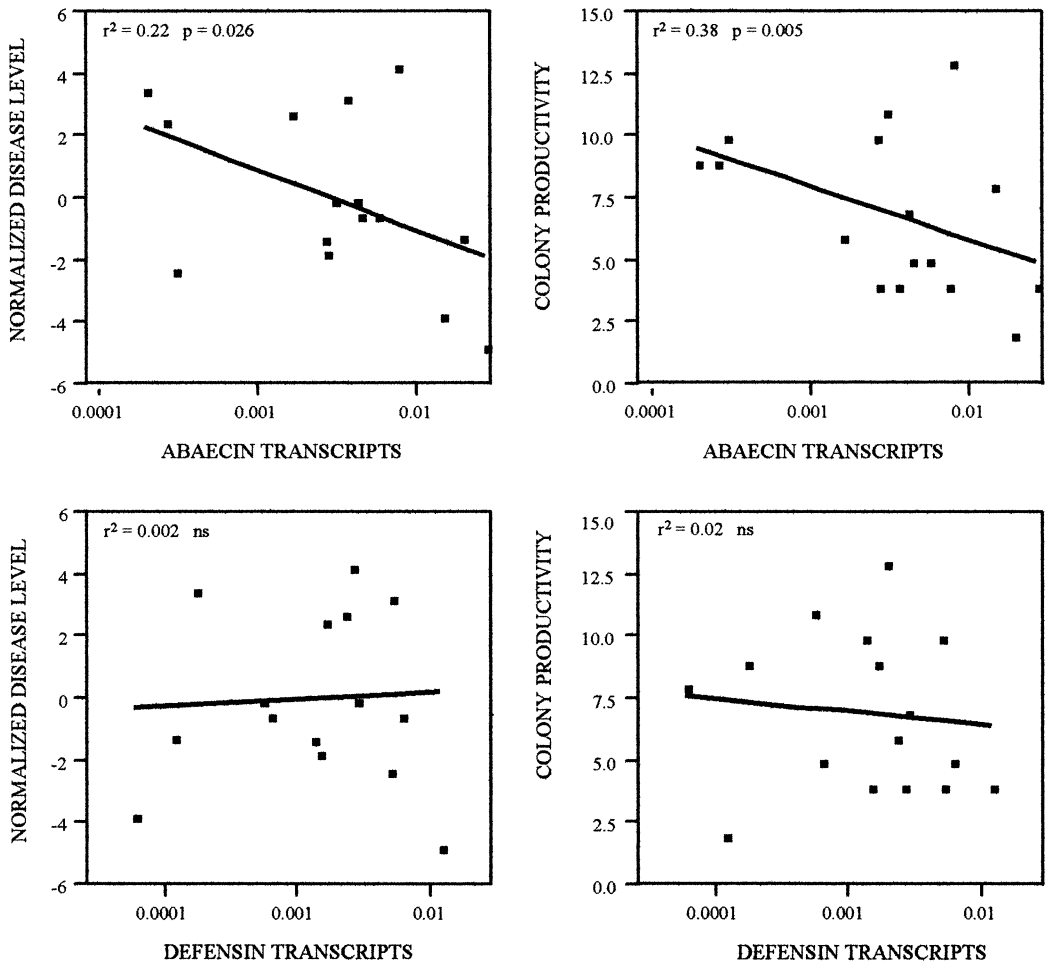

FIG. 1. Relationship between transcript levels for the immunopeptides abaecin and defensin and colony-level American foulbrood disease and productivity. Transcript levels are relative to the housekeeping gene RPS5, and are shown in a $\log_{10}$ scale. Statistics are from two-way ANOVA, as described in text.

veloping brood) as cofactors and immune-transcript levels as dependent variables. Average target gene expression was also compared between colonies with low levels of residual disease ($n = 11$) and those with high disease levels ($n = 5$). The arbitrary cutoff between these two classes was based on previous measures of colonies that had an unsustainable (high) disease level versus those in which colonies could remain viable. All statistical analyses were carried out on C_T differences (e.g., $\log_2$ of transcript copy numbers) since these values followed a normal distribution. Log-transformed (biologically relevant) means consequently have an associated standard error that is asymmetrical (lower SE are smaller than upper SE).

RESULTS

Levels of disease varied substantially across colonies, scaling from no disease present to 26 on the standardized disease score (median disease level = 11). Colonies also differed significantly from each other in larval production levels, ranging from two to 13 frames of developing brood when surveyed (median = 4). There was also substantial variation within and between colonies in induced immune responses. Colony means for transcript levels of the immune-peptide gene abaecin ranged from 1.9×10^{-4} (scaled relative to the control gene RPS5; lower SE = 1.2×10^{-4}, upper SE = 3.8×10^{-4}) to 2.8×10^{-2} (lower SE = 2.1×10^{-2}, upper SE = 7.1×10^{-2}), whereas colony-mean transcripts for defensin ranged from 4.2×10^{-5} (lower SE = 3.2×10^{-5}, upper SE = 9.2×10^{-5}) to 1.2×10^{-2} (lower SE = 9.3×10^{-3}, upper SE = 3.9×10^{-2}).

Abaecin transcript levels in challenged larvae were negatively correlated with both colony-level disease symptoms and colony productivity (Fig. 1a,b). A two-way analysis of variance (model fit $r^2 = 0.60$, $n = 15$ colonies) indicates that abaecin transcript levels are more strongly correlated with productivity (SS = 35.1, F ratio = 11.458, $P = 0.005$) than with colony disease level (SS = 19.9, F ratio = 6.5, $P = 0.026$). Transcript levels of the antimicrobial peptide defensin showed no significant relationship with either productivity or disease (model $r^2 < 0.05$; P values for each cofactor in the ANOVA > 0.5, Fig. 1c,d, Fig. 2b).

When colonies were divided on the basis of measured disease levels ($n = 11$ low-disease colonies, $n = 5$ high disease colonies) larvae from those with higher disease showed lower

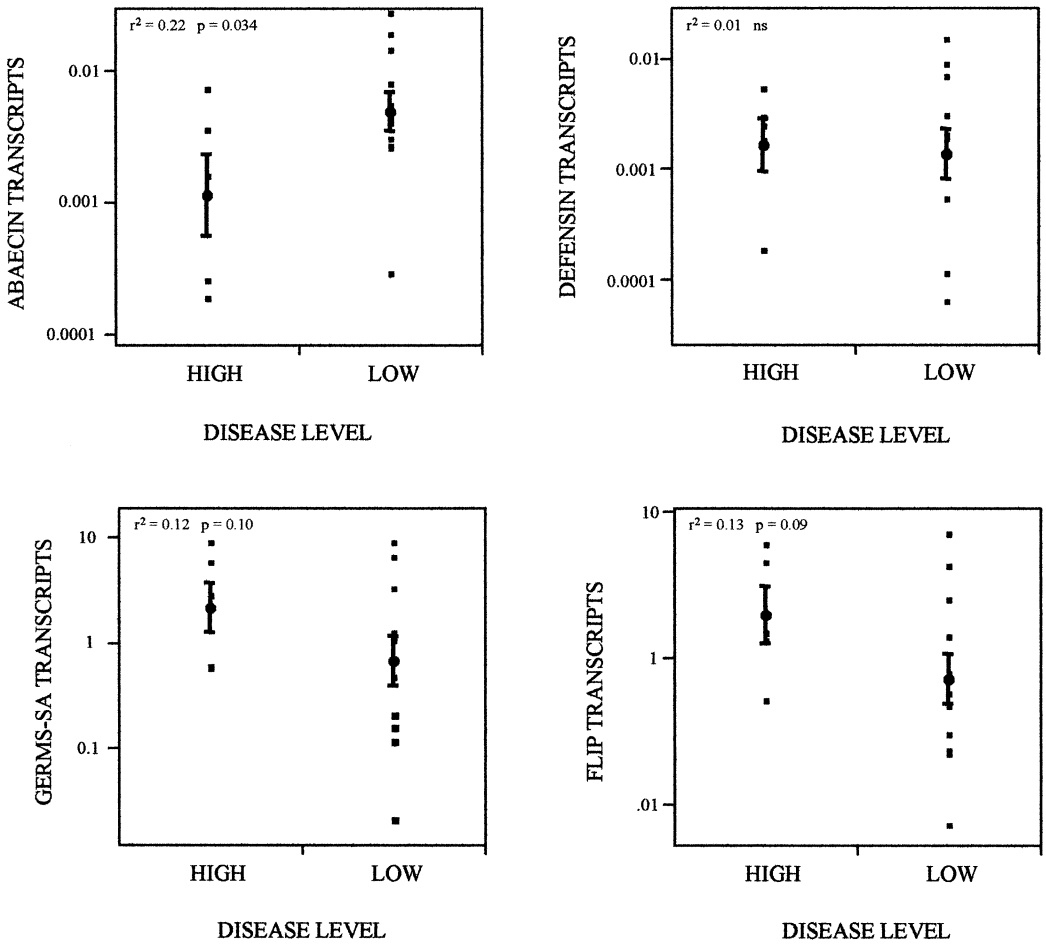

FIG. 2. Transcript levels of immunopeptides abaecin and defensin, and of pathogen genes spore-germination SA and flagellar biosynthesis FliP, in challenged larvae from colonies with low ($n = 11$) or high ($n = 5$) levels of the American foulbrood disease. Bacterial transcripts are normalized to their mean across all colonies, whereas honey bee transcripts are normalized to the housekeeping gene RPS5.

abaecin transcript levels (one-tailed *t*-test, $P < 0.034$). Challenged bees from colonies with high levels of disease also showed a trend toward higher transcript levels for the two developmental genes from their bacterial pathogen (Fig. 2; one-tailed *t*-test, $P = 0.103$ for GermSA, 0.087 for FLiP).

DISCUSSION

We found considerable variation across honey bee colonies in their abilities to survive exposure to a widespread bacterial pathogen. Members of colonies with low disease levels tended to have higher transcript levels for the gene encoding a known antibacterial peptide, abaecin, when compared to colonies with high disease levels. Transcripts for a second antibacterial peptide, defensin, did not covary with disease levels. Abaecin, and not defensin, has been shown previously to be upregulated in response to exposure to *P. l. larvae* (Evans 2004). Transcript levels for two developmental genes from the bacterial pathogen, a germination protein and a flagellar biosynthesis protein, were somewhat higher in challenged bees collected from colonies with a high disease level.

The results suggest a mechanism underlying observed differences across larvae in their tolerance of exposure to *P. l. larvae* (Palmer and Oldroyd 2003). Although honey bees can lower disease levels through nest hygiene and the removal of diseased nestmates, the effect to which larval resistance can impact colony-level disease has been unclear. *Paenibacillus l. larvae* replicates rapidly in larvae as they succumb to foulbrood disease, leaving a larval carcass with $\sim 2 \times 10^9$ infective bacterial spores (Shimanuki 1997). Larvae that avoid this fate by slowing bacterial growth presumably are far less likely to act as sources of spores for developing nestmates.

Colonies whose members mounted a strong immune response tended to have significantly lower larval production rates, indicating a substantial cost to this immune response. Antimicrobial peptides are predicted to be costly to produce (Zasloff 1992). It is possible that larvae producing higher levels of these peptides either require more food during development (a direct energetic cost), survive less well, or develop more slowly. In addition, costs from high immune responsiveness might be expressed during adulthood in the form of worker bees with lowered survival or productivity. Further tests are needed to distinguish between these possible explanations. Regardless, genetic variation in immune responsiveness in bees seems likely to reflect alternate strategies within populations, with some individuals maintaining

and using an expensive immune response upon exposure to bacterial pathogens and others foregoing this response, thereby gaining a productivity benefit when disease exposure rates are low.

We cannot rule out a coevolutionary arms race between bees and this bacterial pathogen as a driving force behind the observed variation across bees in immune traits. Such an arms race seems to explain intercolonial differences in the susceptibility of honey bees to a fungal parasite (Tarpy 2003) and of bumblebees to a parasitic trypanosome (Schmid-Hempel 2004). A more complete experiment involving diverse pathogens and bee lineages is needed to assess the degree, if any, of host-pathogen covariation in disease impacts in this system. Nevertheless, such covariation seems unlikely to explain the observed correlations. First, we used distinct bacterial isolates for our field inoculation and laboratory assays, one from an amalgam of spores collected locally from 100 diseased bees and one derived from an infected colony in California (2500 km distant). Second, such covariation seems unlikely to explain the relationship between colony productivity and immune responsiveness.

Finally, the observed correlation between immune responsiveness, disease levels, and colony productivity could reflect an unmeasured environmental variable that would link these traits. For one, nonpathogenic bacteria found in bees seem to modulate both honey bee immune responses and the growth of the tested pathogen (Evans and Lopez 2004; JDE, unpubl. data). Alternately, prior exposure could lead to a socially mediated protective response toward pathogens arriving later, as shown recently in termites (Traniello et al. 2002). Prior exposure to *P. larvae* or another pathogen could also affect the immune response directly, perhaps even across generations (as demonstrated for an unrelated stimulus in bumble bees; Moret and Schmid-Hempel 2001). Although potentially important, these environmental phenomena are less likely to explain the current results because the tested colonies were recently established in identical hive materials within the same apiary and showed no prior signs of disease. Nevertheless, it will be interesting to explore the combined impacts of both environmental and genetic components on honey bee disease levels and immune responsiveness.

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